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Metabolic stress during winter increases the toxicity of selenium to fish

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This study examined the effect of reductions in water temperature and photoperiod, mimicking winter conditions, on the toxicity of combined dietary (5.1 μg/g dry weight) and waterborne (4.8 μg/l) selenium to juvenile bluegill (*Lepomis macrochirus*). Elevated selenium caused hematological changes and gill damage that reduced respiratory capacity, while increasing respiratory demand and oxygen consumption. Elevated selenium in combination with low water temperature (4°C) caused reduced activity and feeding, depletion of 50–80% of body lipid, and significant mortality within 60 days. Fish in warm-water selenium exposures continued to actively feed and lipid depletion did not occur despite increased oxygen consumption. The combination of stress-related elevation in energy demand and reductions in feeding due to cold temperature and short photoperiod, leading to severe depletion of stored body lipid, is given the name Winter Stress Syndrome. This syndrome caused bluegill to undergo an energetic drain that resulted in death of about one-third of the fish. Results indicate that the current US national water quality criterion for selenium is not adequate to protect young bluegill and other species that substantially reduce activity and feeding during cold weather. Aquatic contaminants should be evaluated in the context of seasonal metabolic changes that normally occur in test organisms. Winter Stress Syndrome could be an important, but as yet unquantified mortality factor in many circumstances.

Key words: Selenium; Bluegill; Centrarchids; Winter mortality; Metabolic stress

INTRODUCTION

Selenium is a trace element that is normally present in surface waters at concentrations of about 0.1–0.3 μg/l (parts-per-billion; Lemly, 1985a). In slightly greater amounts, i.e., 1–5 μg/l, it can bioaccumulate in aquatic food-chains and become a concentrated dietary source of selenium that is highly toxic to fish and wildlife (Lemly and Smith, 1987; Lemly, 1993a). Dietary selenium is passed from parents to offspring

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in the eggs, where it can be teratogenic to developing embryos and cause complete reproductive failure (Gillespie and Baumann, 1986; Heinz et al., 1987, 1989; Coyle et al., 1993; Lemly, 1993b). This scenario of poisoning has occurred in reservoirs contaminated by selenium leached from fly-ash at coal-fired electric generating stations in the eastern US (Garrett and Inman, 1984; Lemly, 1985b), and in wetlands used to dispose subsurface agricultural irrigation drainage in the western US (Lemly, 1993c; Lemly et al., 1993). Reports of selenium accumulation and reproductive failure in fish populations are mostly from aquatic habitats where warm-water sport fish such as centrarchids (largemouth bass, bluegill, other sunfish), percichthids (white bass and striped bass), and ictalurids (catfish) predominate. The margin of safety for selenium – i.e., the difference between normal waterborne concentrations and those that lead to substantial bioaccumulation and reproductive failure – is extremely small, and impacts on warm-water fish populations may become dramatic with only a slight increase in waterborne selenium (Lemly, 1985a).

Because of the population and ecosystem-level implications of reduced reproductive success, it is important to know what levels of selenium produce reproductive impairment in fish. Concentrations greater than 10 μg/g (parts-per-million) dry weight usually result in partial or complete reproductive failure (Gillespie and Baumann, 1986; Lemly, 1993a). However, fish that are not in reproductive condition or have not reached reproductive age will not be evaluated using this type of assessment even though it is desirable to determine the selenium status and health of these two sectors of the population. Moreover, it is important to know the amount of non-reproductive mortality, i.e., the direct effects on juvenile and adult fish, in order to accurately assess the total impact of selenium toxicity on fish communities. Observations made at power plant cooling reservoirs indicate that substantial non-reproductive mortality occurs during the early stages of selenium contamination, but the specific cause(s) have not been determined (Harrell et al., 1978; Bryson et al., 1984; Garrett and Inman, 1984).

Field studies of contaminated aquatic ecosystems have shown that several histological and hematological parameters change in adult centrarchids exposed to elevated selenium in the water and diet. These responses involve tissue damage in major organs such as the liver and kidney, reduced weight and body condition factor, and reduced numbers and size of red blood cells (Sorensen, 1986, 1988; Sorensen and Bauer, 1983, 1984a,b; Sorensen et al., 1982a,b, 1983a,b, 1984). Although these findings indicate a strong link between environmental exposure and tissue pathology in adult fish, it is not known whether juveniles experience similar effects or whether the conditions eventually lead to mortality. The pathological changes were assumed to be detrimental to the health of the fish but no experimental studies were conducted to show whether or not survival was affected. Thus, the importance of direct mortality associated with tissue damage relative to the indirect effects of selenium on reproductive success is not clear. It is possible that moderate damage to some tissues could be tolerated indefinitely through a compensatory mechanism, thus having little biologi-

cal significance, while for other tissues and organs there could be a temporal limit beyond which mortality occurs.

In order to evaluate the importance of non-reproductive mortality in an environmentally relevant context, it is necessary to measure the responses of centrarchids to selenium in exposures that are within or below the range where reproductive effects due to bioaccumulation first begin to occur, i.e., 2–5 μg/l in water and 5–10 μg/g in the diet. At greater concentrations, reproductive failure is so severe that entire fish communities may be eliminated (Cumbie and Van Horn, 1978; Lemly, 1985b). Non-reproductive mortality, therefore, becomes insignificant as the level of selenium contamination rises. However, many aquatic habitats could experience small increases in selenium from a variety of industrial and agricultural sources (Hodson et al., 1984; Lemly, 1985a, 1993; Lemly et al., 1993). In these situations it is important to know the population and community-level implications of concentrations on the low end of the spectrum. Gradual increases of 1–2 μg/l selenium could lead to subtle, but significant mortality among juvenile and adult fish that would go undetected if reproductive endpoints alone were used to make an environmental assessment.

Results from laboratory studies suggest that concentrations of 2–5 μg/l selenium in water and 5–10 μg/g in the diet are safe for juvenile centrarchids (Adams, 1976; Lemly, 1982; US Department of the Interior, 1990). However, the tests did not mimic environmental conditions with regard to seasonal changes in water temperature, most importantly, the onset of winter. Cold water temperatures depress feeding activity in juvenile centrarchids and can cause stressors that are normally tolerated during warm weather and active feeding to become lethal. The mechanism underlying this phenomenon involves a depletion of stored body fat necessary for winter survival. As water temperatures drop, the fish reduce feeding but if the stressor remains in place, elevated metabolic demands continue. The result is that lipid reserves are sharply reduced and are insufficient to fuel the body through winter; mortality then occurs (Lemly, 1980; Lemly and Esch, 1984). Adult centrarchids feed more actively than juveniles during winter and are not as susceptable to this type of nutritional distress and associated mortality.

The present study was conducted to determine effects of selenium on juvenile centrarchids in exposures that resemble the early stages of contamination in natural aquatic systems. The hypothesis tested in this investigation was that the tissue damage observed for centrarchids exposed to elevated selenium constitutes a physiological stressor that, when combined with the challenge of winter conditions, reduces survival. The strong correlation observed between elevated tissue concentrations of selenium and low body condition of centrarchids in the field (Sorensen and Bauer, 1984b) provides evidence of metabolic stress. Further, if this stressor operates like the host–parasite interactions described by Lemly and Esch (1984), there should be a reduction in lipid reserves during winter, possibly accompanied by mortality of juveniles.

Specific objectives of the study were to: (i) determine the histological and metabolic response of juvenile bluegill simultaneously exposed to 5 μg/l selenium in water and

5 μg/g in the diet over a period of 6 months, during which temperatures were lowered and photoperiod shortened to mimic winter conditions, and (ii) to determine effects of selenium on the survival of these fish and identify causes for any mortality that occurred. Using this approach, impacts of selenium on bluegill were defined in terms of how seasonal variation in environmental factors affect toxicity. Moreover, the potential effects of selenium on the non-reproductive sector of a bluegill population were determined for the 'gray area' where selenium concentrations are below levels that cause catastrophic poisoning, i.e., where some reproduction is occurring but the population is still affected.

MATERIALS AND METHODS

Experimental animals and exposures

Juvenile bluegill (*Lepomis macrochirus*) were caught with seines from ponds having selenium concentrations ≤ 0.15 μg/l and a water hardness of 15–25 mg/l as $CaCO_3$. Collections were made during late summer and individuals 50–70 mm total length were returned to the laboratory and held in 200-l glass aquaria for observation to ensure that only healthy fish were used in the tests. Eight groups (70 fish per group) were separated and placed into individual 400-l fiberglass tanks which served as the testing chambers. Six of the groups were exposed to combined waterborne (5 μg/l) and dietary (5 μg/g dry weight) selenium for a period of 180 days; three groups in warm water (20°C) and three in cold water (4°C). The remaining two groups served as both temperature and selenium controls and were held in low-selenium water (one group in warm water, one in cold) and fed an uncontaminated diet.

Selenium used for the waterborne component of exposures was formulated using a 1:1 mix of selenate (Na_2SeO_4) and selenite (Na_2SeO_3; analytical grade, Aldrich Chemical Co.). This ratio was chosen because it represents the relative amounts of inorganic selenium forms encountered in power plant cooling reservoirs (Cutter, 1986, 1988). Aqueous stock solutions were prepared in de-ionized water and adjusted to pH 2 with analytical grade HCl to prevent bacterial growth and changes in the oxidation state of the selenium constituents. This mixture was dosed into the exposure tanks using an intermittent-flow dilution apparatus as described by McAllister et al. (1972) and Chandler et al. (1974). The intended concentration of dissolved selenium (5 μg/l) was checked daily by determing the ratio between flow of stock solution and flow of dilution water. Samples from each treatment and control were analyzed weekly to verify the actual concentrations of waterborne selenium present and to evaluate the performance of the dosing equipment. Diluent water consisted of aerated (24 h) tap water with the following basic chemistry: total hardness, 20–25 mg/l as $CaCO_3$; dissolved oxygen content, 8.1–8.6 mg/l; pH, 6.1–6.6; residual chlorine, < 3 μg/l; total ammonia, < 0.01 mg/l; dissolved selenium content, ≤ 0.15 μg/l. Flow rates were adjusted to provide one volume replacement per day in the tanks.

Seleno-L-methionine (Sigma Chemical Co.) was used as the source of selenium in the dietary component of exposures. This chemical form of selenium is a good experimental model for the organic form(s) of selenium that occur naturally in aquatic food-chains in the field (Hamilton et al., 1986). TetraMin® flake food was homogenized to produce a base diet and then an aqueous solution of seleno-L-methionine was added to yield a nominal selenium concentration of 4.0 μg/g wet weight. Average moisture content of the preparation was 25%, resulting in a nominal total selenium concentration of 5 μg/g dry weight. Control diet consisted of homogenized TetraMin with the same moisture content but without added seleno-L-methionine. All diets were pelletized, vacuum-packed in plastic bags containing about 500 g each, and frozen at −30°C. Actual concentrations of selenium were determined for three samples of control and experimental diets at the begining and end of the tests. The fish were fed each morning at 08:00 h at a rate of 3% body weight per day, based on the average weight per fish calculated at the begining and mid-point of the study. Fecal material and uneaten food were siphoned from the tanks daily. The volume of uneaten food was measured and compared among treatments, and between treatment and control tanks.

During the experiments, water temperature was manipulated with submersible heaters and chilling units. Exposures began at 20°C and in half of the experimental groups (one control and three treatment replicates), temperature was decreased at a rate of 2°C per week for 8 weeks in order to mimic the onset of winter conditions. Once the minimum temperature was reached (4°C) it was maintained for the remainder of the 180-day exposure. Lighting was supplied from a bank of incandescent and fluorescent bulbs that produced an intensity of 70 foot-candles at the water surface. The lights were controlled with timers such that the incandescent bulbs were illuminated 15 min prior to the fluorescent bulbs each morning. The exposures began with a 16:10 h light/dark photoperiod which was gradually reversed to a 10:16 h light/dark cycle by day 60 of the tests. Reductions in photoperiod coincided with reductions in water temperature and were done to further simulate winter conditions. Temperature and photoperiod were held constant throughout the 180-day test for the warm treatment and control (20°C; 12:12 h light/dark cycle). Thus, controls were included for both temperature and selenium exposure. Mortality was recorded for each tank daily and body condition was calculated for all dead fish.

Histological, hematological and metabolic measurements

Ten fish were removed at random from each treatment replicate and control on days 0, 60, 120, and 180, anesthetized, and killed by severing the spine. The fish were weighed and body condition was determined based on the formula:

$$K = \frac{100 \times \text{weight (g)}}{\text{standard length}}$$

and was considered to reflect the general state of health (Bagenal, 1978). Five of these fish were used to make a composite sample for determining whole-body selenium concentrations and lipid content, and the other five were dissected to determine histopathological effects of selenium exposure. The composite tissue samples were refrigerated at 4°C until lipid and selenium determinations were made, usually within 7 days but never more than 14 days later. Samples of liver, kidney, gill, heart, and spleen were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned and stained with hematoxylin and eosin for examination by light microscopy.

Blood was sampled by removing the caudal fin and filling heparinized capillary tubes directly from the caudal vessels. These samples were used for measuring hematocrit and hemoglobin concentration, and making blood smears for erythrocyte and leukocyte counts. Blood smears were fixed in methanol and stained with Giemsa before making cell counts. Erythrocyte–leukocyte differentials were made by counting 100 cells in each of three areas on a blood smear (total of 300 cells per smear). The percentage of mature erythrocytes, erythroblasts (immature red cells), nuclear shadows (disintegrated nuclei from old or fragile erythrocytes; Yuki, 1960), neutrophils, and lymphocytes was determined.

Hemoglobin concentration was determined with a spectrophotometer using the cyanmethemoglobin procedure (Dacie and Lewis, 1975). Mean corpuscular hemoglobin concentration (MCHC) was calculated using the expression:

$$\text{MCHC} = \frac{\text{hemoglobin (g/100cc blood)} \times 100}{\text{hematocrit (\%)}}$$

This measurement indicates the relative hemoglobin saturation of erythrocytes and can be used to identify the presence of anemia (Best, 1949). Mean corpuscular volume (MCV) was calculated as follows:

$$\text{MCV} = \frac{\text{hematocrit (\%)} \times 10}{\text{red cell count } (10^6\text{ccm})}$$

This term quantitatively estimates the average size of individual erythrocytes and can thus detect the presence of unusually small (microcytic), large (macrocytic), or deformed (poikilocytic) cells, all of which may occur in response to environmental contaminants (Nikinmaa, 1992).

Metabolic status was determined by measuring lipid content in composite, whole-body fish samples. Two types of samples were prepared; composites of five individuals removed from each treatment replicate and control on days 0, 60, 120 and 180, and treatment-specific composites of fish that died during the study. Dead fish were frozen at −30°C and sorted by time interval (0–60 d, 61–120 d, 121–180 d). Five individuals from each interval, or as many as were available if fewer than five died, were used to formulate the composite sample (half of each sample was analyzed for selenium concentration). Homogenized samples were mixed in a solution of hexanes and the extracted lipids were decanted, air dryed, and weighed. The procedure is

described in detail by Bligh and Dyer (1959). Because the tissue samples were composites, lipid values represent averages and are expressed as percent body weight.

Oxygen consumption was determined for five fish randomly selected from each treatment replicate and control on day 60, 120, and 180 (± 1 day). This measurement was made to provide an estimate of the metabolic demands, i.e., metabolic stress, being experienced by the fish, as indicated by level of activity and respiration (Lemly and Esch, 1984). A 1.0 liter plexiglass box was used as a test chamber. Experiments began with air-saturated water, and oxygen concentrations in the water were monitored with a YSI Model 54320 stirring polarographic oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH). A fish was placed in a small cage in the test chamber to provide visual isolation and reduce fright behavior. The chamber was sealed, the oxygen probe was inserted, and the stirring mechanism was engaged. The system was allowed to equilibrate for 30 s, after which the concentrations of dissolved oxygen were monitored for 15 min. Following the procedure, fish were returned to their respective tanks. The rate of oxygen consumption (QO_2, in mg/kg/h) was subsequently calculated and compared between treatments and their corresponding control.

Determination of selenium concentrations

All selenium measurements were made using hydride generation techniques with a Varian SpectrAA-20 atomic absorption spectrophotometer equipped with a Varian PSC-56 autosampler and VGA-76 hydride generator. Fish tissue and food were freeze-dried with a Virtis SRC-4X sublimator, and moisture content was determined by difference between wet and dry weight. Dried samples were blended and prepared for analysis using a dry-ash oxidative fusion technique (May, 1982; Hansson et al., 1987). After the oxidation step, samples were treated with hot hydrochloric acid to convert all selenium present to the selenite form. Water samples were prepared for analysis using a two step persulfate oxidation/hydrochloric acid reduction procedure (Ingersoll et al., 1990).

Four types of samples were used to assess quality control in selenium determinations; procedural blanks, pre- and post-digestion spikes, replicate analyses, and reference materials with known selenium content. Limits of detection averaged 0.070 μg/g Se for tissue and food, and 0.0056 μg/l Se for water. All reagent blanks had selenium concentrations below the limits of detection. Recovery of selenium spikes ranged from 83–112% for pre-digestion samples (average = 94%), and 88–105% for post-digestion samples (average = 97%). The mean relative percent difference of duplicate analyses was 3.5 (n = 28). The selenium concentration determined for National Bureau of Standards RM 50 (Albacore Tuna) consistently fell within the certified acceptable range.

Statistical analysis of data

Data analyses were performed on a personal computer using Statistical Analysis System (SAS) software programs (SAS Institute, Cary, NC). Comparisons of body condition, lipid content, oxygen consumption, and survival were made with split-plot analysis of variance using selenium exposure and water temperature as main effects (Sokal and Rohlf, 1981). Differences between means were tested with least-square means comparison. Statements of significance indicate that statistical probabilities were $\leq 5\%$.

RESULTS

Selenium concentrations

Measured mean concentrations of selenium in the treatments were 5.16 μg/g dry weight in the diet (range = 5.01–5.33, $n = 6$) and 4.83 μg/l in the water (range = 4.49–

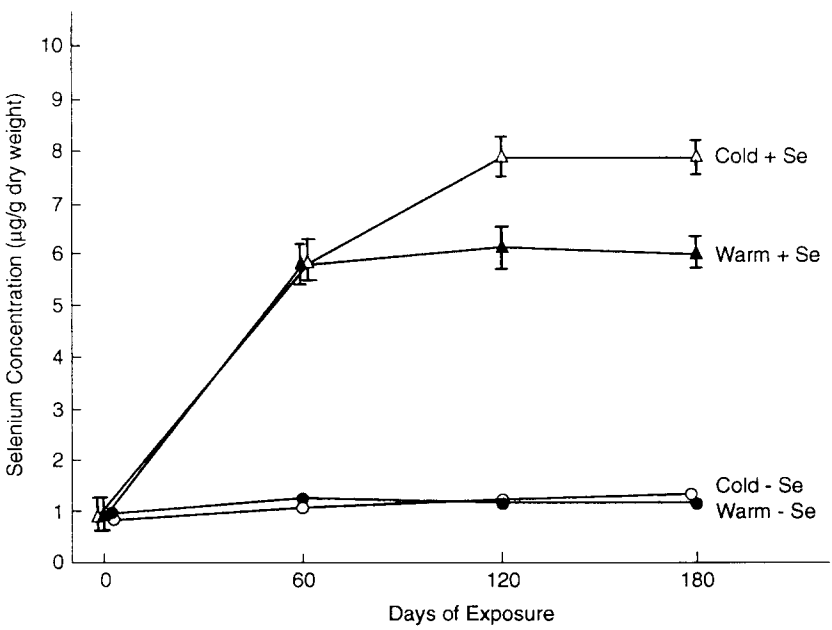

Fig. 1. Whole-body concentrations of selenium in juvenile bluegill exposed to combined waterborne and dietary sources. Each data point represents composite samples made from five fish; $n = 1$ for controls (Warm–Se, Cold–Se), $n = 3$ for treatments (Warm + Se, Cold + Se), with the geometric mean and range reported. Water temperature was held constant at 20°C throughout the study for exposures designated as 'Warm'; temperature was decreased 2°C per week for 8 weeks and then held constant at 4°C in exposures denoted 'Cold'. Treatments identified as '+Se' received 5.1 μg/g dry weight selenium in the diet and 4.8 μg/l in water; '−Se' indicates selenium controls, which received 0.8 μg/g in diet and 0.16 μg/l in water.

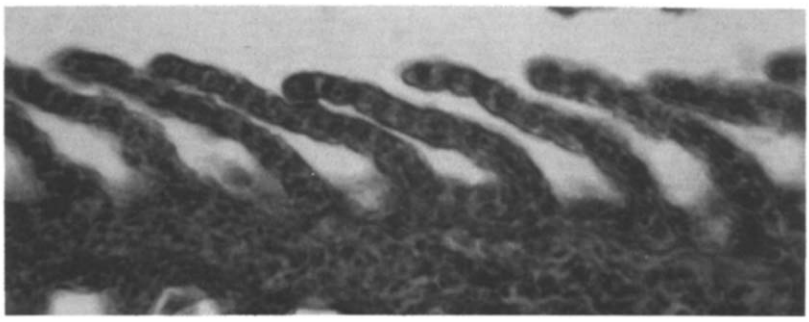

Fig. 2. A section of gill tissue of a fish from the cold-water control. Secondary lamellae are uniform and well defined, with no evidence of swelling or other pathological conditions. Paraffin-embedded, hematoxylin and eosin stained, 240 ×.

5.12, $n = 150$). Concentrations for the control groups were 0.82 μg/g in the diet (range = 0.74–0.89, $n = 6$) and 0.159 μg/l in the water (range = 0.145–0.165, $n = 50$). There was no storage-related change in selenium content of the rations fed during the study. Whole-body selenium concentrations in bluegill were elevated in all of the selenium treatments on day 60 of the study (Fig. 1). Concentrations on day 60 were 5–6 μg/g dry weight, which was approximately the same as those in the diet. On day 120, concentrations of selenium remained elevated in all selenium treatments and were significantly greater in the cold-water treatment than in the warm-water treatment. Concentrations in the cold-water fish were 7–8 μg/g, or about 30% greater than in the warm-water fish. This pattern of selenium bioaccumulation persisted through day 180 of the study (Fig. 1). The concentration of selenium in fish that died during

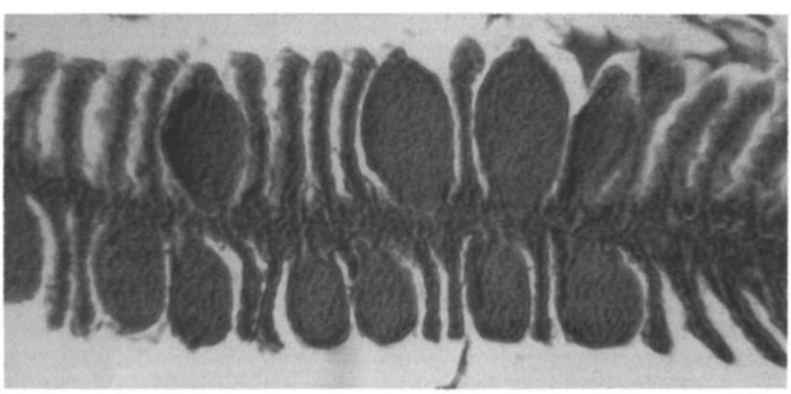

Fig. 3. Gill filament of a fish taken from the warm-water selenium treatment on day 120. Severe swelling, or telangiectasia, of the blood sinuses is present in several of the secondary lamellae. Paraffin-embedded, hematoxylin and eosin stained, 220 ×.

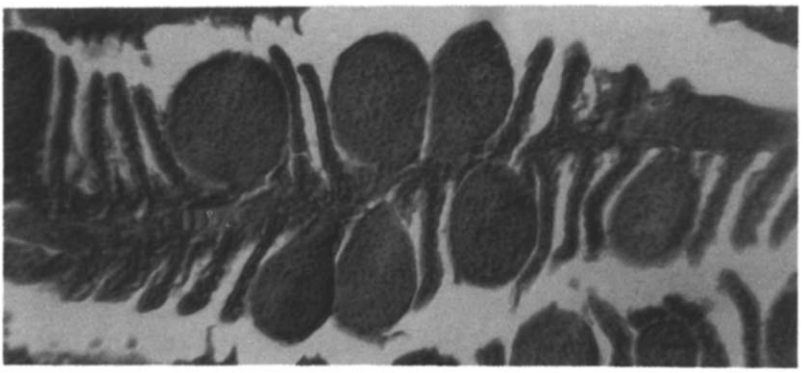

Fig. 4. Gill tissue of a fish taken from the cold-water selenium treatment on day 120. Telangiectasia of blood sinuses affected up to 100% of the secondary lamellae and as many as 60% were severely distorted. The occurrence of telangiectasia was similar both in the cold- and warm-water selenium treatments. Paraffin-embedded, hematoxylin and eosin stained, 220 ×.

the experiments was not statistically different from that of live fish collected from the corresponding tank on designated sampling days, i.e., day 60, 120, and 180.

Tissue pathology

No gross or microscopic abnormalities were detected in liver, kidney, heart, or spleen for any of the treatments or controls. The only pathological condition observed was a change in the structure of gill tissue in the fish exposed to elevated selenium. This change involved a marked swelling of blood sinuses in the secondary lamellae, a condition known as telangiectasia (Figs. 2–5). Little swelling was present

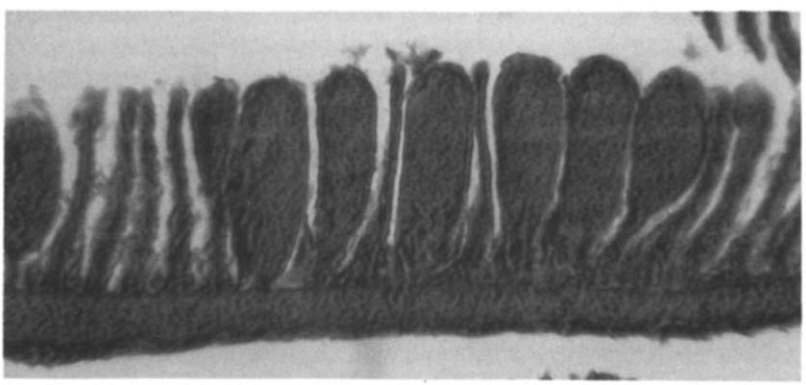

Fig. 5. Tangential section of a gill filament of a fish taken from the cold-water selenium treatment on day 180, the end of the study. Severe telangiectasia persists in a large number of secondary lamellae. Paraffin-embedded, hematoxylin and eosin stained, 220 ×.

at day 60 of the exposures, but was severe on day 120 and 180. As many as 100% of the lamellae exhibited some swelling and up to 60% were severely distended. Gill damage occurred in both the warm- and cold-water selenium treatments and persisted through the end of the study.

Hematology

Hematocrit, mean corpuscular hemoglobin concentration, mean corpuscular volume, and percentage of erythroblasts were all significantly lower in selenium treatments than in controls (Table 1). The percentage of mature erythrocytes and number of nuclear shadows was significantly higher in the selenium-exposed fish. Effects were consistent in cold-water and warm-water fish, and occurred to the same extent on days 60, 120, and 180 of the study. The numbers of lymphocytes and neutrophils did not differ significantly between treatments and controls.

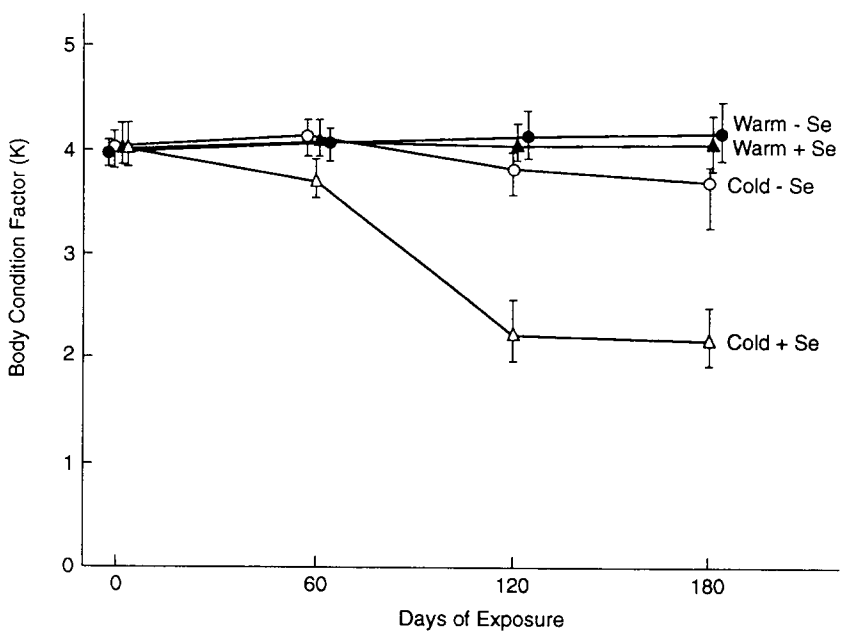

Fig. 6. Body condition (K) of bluegill exposed to elevated dietary (5.1 μg/g dry weight) and waterborne (4.8 μg/l) selenium. Values are the geometric mean and range for ten fish in the controls (Warm–Se, Cold–Se), and 30 fish in the treatments (Warm + Se, Cold + Se). Condition was significantly lower in the cold-water treatment on days 120 and 180.

TABLE 1
Effects of selenium exposure and water temperature on hematological values in bluegill (*Lepomis macrochirus*).

Exposure conditions and blood parameter[a]	Day of study			
	0	60	120	180
Warm water, low selenium[b]				
Total erythrocytes (10^6/ml)	2.95	2.96	2.99	2.96
% mature	85	86	86	85
nuclear shadows (10^4/ml)	0.95	0.97	0.83	0.91
Total leukocytes (10^4/ml)	17.22	16.90	16.73	17.05
% lymphocytes	23	20	19	21
% neutrophils	15	14	17	17
Hematocrit (%)	37	37	36	38
MCHC[c]	23	25	25	25
MCV[d]	177	172	180	169
Warm water, high selenium[e]				
Total erythrocytes	2.92	2.93	2.95	2.89
% mature	86	93*	94*	94*
nuclear shadows	0.86	2.05*	2.38*	2.30*
Total leukocytes	17.41	17.55	17.62	17.36
% lymphocytes	25	23	26	22
% neutrophils	13	15	19	16
Hematocrit	36	29*	29*	28*
MCHC	25	19*	18*	17*
MCV	176	150*	142*	141*

Body condition and feeding

Condition factors were lower for the cold-water, selenium-exposed fish than for the other treatment and controls (Fig. 6). Small changes were apparent by day 60 of the study and significant reductions were present on days 120 and 180. There was no difference in condition between the warm-water selenium treatment and warm- or cold-water controls. Qualitative visual estimates indicated that the feeding activity of fish in the cold-water treatment and control was depressed by day 60 of the study, which represented the period of declining water temperatures. Measurements of the volume of uneaten food siphoned from tanks each day indicated that by day 120 the cold-water fish were eating less than 10% of the amount consumed by warm-water fish. No differences in feeding activity or food consumption were noted between the cold-water control and cold-water selenium treatment, or between the warm-water control and treatment. The body condition of fish that died during the experiments was not significantly different than that of live fish from the corresponding tank.

TABLE 1 (continued)

Exposure conditions and blood parameter[a]	Day of study			
	0	60	120	180
Cold water, low selenium[f]				
Total erythrocytes	2.91	2.97	3.01	3.00
% mature	84	87	85	85
nuclear shadows	0.86	0.83	0.89	0.90
Total leukocytes	16.48	16.79	16.80	16.96
% lymphocytes	17	16	19	19
% neutrophils	13	15	15	12
Hematocrit	39	40	41	39
MCHC	26	25	22	23
MCV	182	188	180	185
Cold water, high selenium[g]				
Total erythrocytes	2.93	2.90	2.95	2.99
% mature	82	95*	96*	97*
nuclear shadows	0.84	2.30*	2.49*	2.36
Total leukocytes	16.88	16.91	16.74	16.63
% lymphocytes	16	17	15	18
% neutrophils	12	11	12	14
Hematocrit	37	30*	28*	27*
MCHC	25	18*	17*	17*
MCV	171	146*	135*	130*

[a] Values for all parameters represent means for five fish.

[b] Water temperature maintained constant at 20°C; 0.8 μg/g selenium in diet, 0.6 μg/l in water.

[c] Mean corpuscular hemoglobin concentration, in g/100 cc packed erythrocytes.

[d] Mean corpuscular volume for red cells, in cubic microns.

[e] Water temperature maintained constant at 20°C; 5.1 μg/g selenium in diet, 4.8 μg/l in water

[f] Water temperature decreased from 20°C to 4°C during first 60 days of study and maintained at 4°C thereafter; 0.8 μg/g selenium in diet, 0.6 μg/l in water.

[g] Water temperature decreased from 20°C to 4°C during first 60 days of study and maintaned at 4°C thereafter; 5.1 μg/g selenium in diet, 4.8 μg/l in water.

* Significantly different from corresponding temperature–selenium control value.

Oxygen consumption

Both selenium and temperature affected the rate of oxygen consumption by bluegill. As water temperatures declined in the cold-water treatment and control, oxygen consumption by these fish decreased until, by day 60, the rate of consumption was significantly lower (Fig. 7). Among treatments and controls with the same temperature, selenium had no effect on oxygen consumption on day 60. However, by day 120, selenium-exposed fish were consuming significantly more oxygen in both the

warm- and cold-water treatments. This trend persisted through the rest of the study. QO_2 was about 15–30% higher in the selenium-exposed fish than in the corresponding control on days 120 and 180.

Lipid content

Total body lipid of bluegill at the begining of the study was 12–14%, and did not change in the warm-water selenium treatment and warm-water control for the remainder of the study. However, large reductions in lipid occurred in the cold-water selenium treatment (Fig. 8). Lipid content in these fish fell to 6–11% by day 60, and was further reduced to 3–8% by day 120; it remained at this level through day 180. Gradual reductions in lipid occurred in the cold-water control fish, but the changes were not as quick or severe as in the cold-water selenium treatment; levels fell to only about 10% by day 180. Lipid content of fish that died during the study averaged 4.3% (range = 3.1–5.9%, n = 11). These lipid values were not different from those of live fish removed from the corresponding tank on scheduled sampling days.

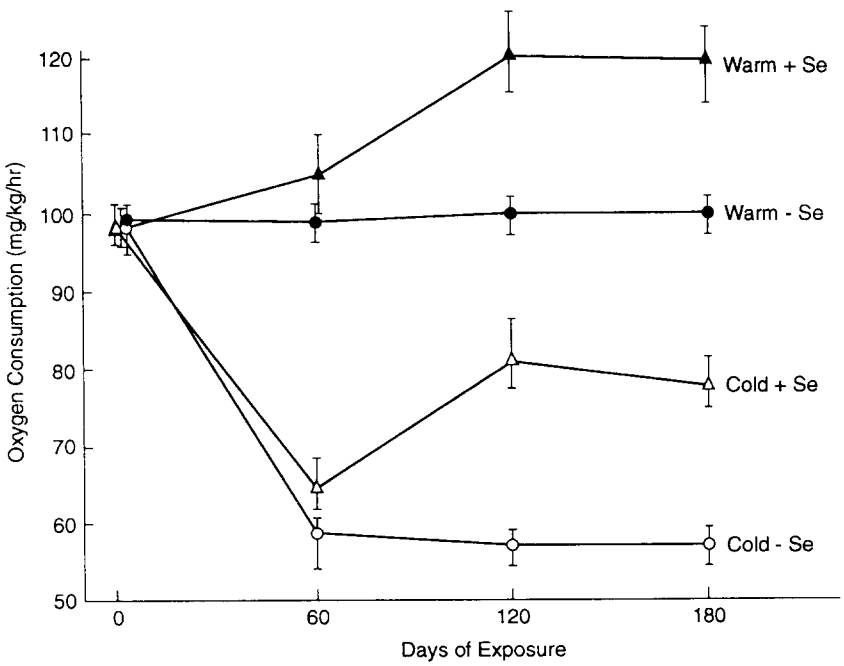

Fig. 7. Oxygen consumption of bluegill exposed to elevated dietary (5.1 μg/g dry weight) and waterborne (4.8 μg/l) selenium. Values are the geometric mean and range for five fish in the controls (Warm–Se, Cold–Se), and fifteen fish in the treatments (Warm + Se, Cold + Se). The rate of consumption was significantly elevated in selenium treatments relative to the corresponding control on days 120 and 180.

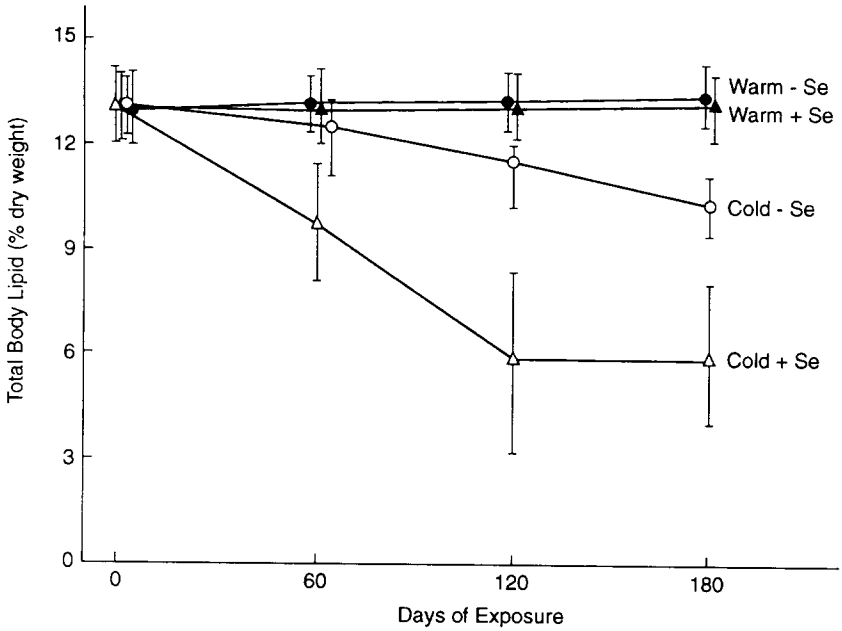

Fig. 8. Lipid content of bluegill exposed to elevated dietary (5.1 μg/g dry weight) and waterborne (4.8 μg/l) selenium. Each data point is the geometric mean and range for composite samples of 2–5 fish; n = 2–3 for controls (Warm–Se, Cold–Se), n = 3–5 for treatments (Warm + Se, Cold + Se). Lipid was an average of 7–9% lower in the cold-water selenium treatment than controls and the warm-water selenium treatment on days 120 and 180.

Mortality

Five fish died in the controls during the study, two in the cold-water group (2.8%) and three in the warm-water group (4.3%). These deaths occurred sporadically during the study and are unaccounted for. Nevertheless, the amount of mortality in controls was well below 10%, which is the maximum level considered acceptable to make valid conclusions based on differential survival in contaminant studies (Rand and Petrocelli, 1985).

Mortality in the warm-water selenium treatment was slightly greater than the control but was not statistically different; a total of 12 fish died in the three replicate tanks (5.8%). However, significant mortality did occur in the cold-water selenium treatment (Fig. 9). On day 60, a total of 15 fish had died in the three replicate tanks (7.0%). This number increased to 62 fish on day 120 (29.5%), and 71 fish on day 180 (33.8%). Total mortality was fairly uniform among the replicates (24, 27, 20), and occurred primarily during the 60–120 day interval in each tank. Assuming that handling stress from oxygen consumption measurements or other factors resulted in control-level mortality in the cold-water selenium treatment (2 dead fish/tank × 3 tanks = 6 fish), approxi-

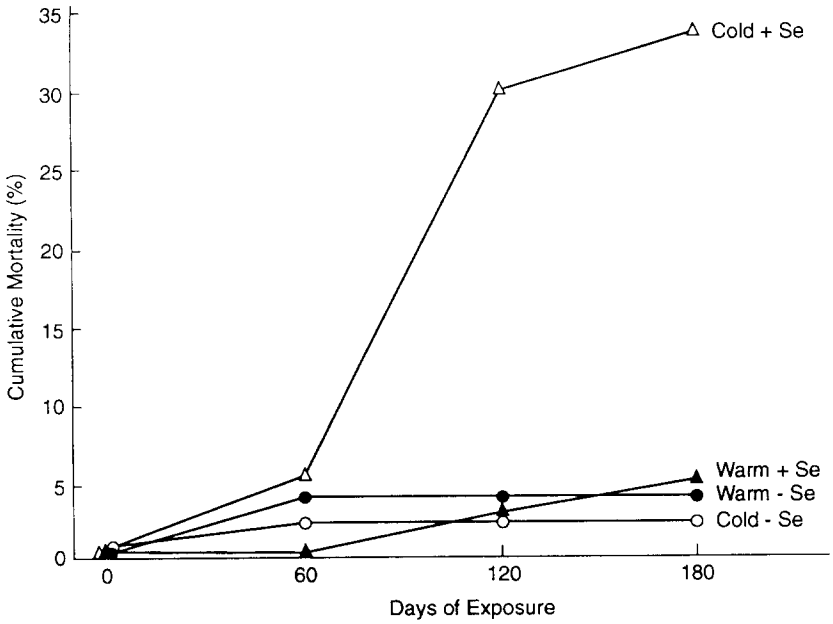

Fig. 9. Cumulative mortality of juvenile bluegill exposed to combined waterborne and dietary selenium. The two selenium treatments, Warm + Se, Cold + Se, had three replicates, each with seventy fish. The two controls, Warm−Se, Cold−Se, also had seventy fish each at the begining of the study. Water temperature was held constant at 20°C throughout the study for exposures designated as 'Warm'; temperature was decreased 2°C per week for 8 weeks and then held constant at 4°C in exposures denoted 'Cold'. Treatments identified as '+Se' received 5.1 μg/g dry weight selenium in the diet and 4.8 μg/l in water; '−Se' indicates selenium controls, which received 0.8 μg/g in diet and 0.16 μg/l in water. Most of the mortality in the Cold−Se treatment took place within about 60 days after water temperature reached its low point of 4°C, which occurred between days 50 and 60.

mately 92% of the treatment effect, i.e., 65 mortalities, is attributable to selenium exposure.

DISCUSSION

Hematological changes and gill damage

The reduction in MCHC which occured in bluegill exposed to elevated selenium in this study indicates that anemia developed, both in the warm- and cold-water treatment. Lowered hemoglobin level has long been recognized as a symptom of selenium-related anemia in vertebrates, including fishes (Franke and Potter, 1934; Ellis et al., 1937). Field studies of centrarchids chronically exposed to elevated selenium in the diet indicate that similar reductions in MCHC occur in a natural setting (Sorensen

and Bauer, 1983). In addition to causing impaired respiration through reductions in MCHC, selenium can apparently bind to hemoglobin, rendering it incapable of carrying oxygen. Acute poisoning and death have been attributed to cellular asphyxiation resulting from selenium inhibition of hemoglobin (Miller and Williams, 1940). In the present study, bluegill developed reduced respiratory capacity by day 60 in the selenium treatments, consisting of significant reductions in MCHC, MCV, and hematocrit (Table 1).

Reduction in the percentage of erythroblasts in selenium-exposed fish suggests that erythropoiesis was negatively affected even though no pathological conditions were detected in the spleen or kidney. Significant increases in the number of nuclear shadows and proportion of mature red cells indicated that the circulating erythrocyte population was aging, and replacement by new cells was slowing. The increase in nuclear shadows may also have been partly due to impaired delivery of oxygen to the erythrocytes themselves, resulting in premature cell death from asphyxiation.

The hematological changes could have been responsible for subsequent gill damage, which was evident on days 120 and 180. This tissue damage occurred in the warm- and cold-water selenium treatment and was characterized by telangiectasia in secondary lamellae (Figs. 2–5). Light microscopy revealed that the swelled and deformed lamellae were packed with erythrocytes and the overall lamellar structure resembled an aneurism. It seems likely that impaired blood flow was responsible for this condition. Nuclear shadows (disintegrated cell nuclei) were significantly elevated in the blood of these fish (Table 1). It is possible that cell parts collected in the capillary beds and restricted blood flow, thereby increasing blood pressure and rupturing or swelling lamellar vessels. Mean corpuscular volume was significantly reduced in selenium-exposed fish and could have added to the problem by causing small red cells to become tightly packed into the vessels as they became dilated.

This report is the first to experimentally confirm that telangiectasia in fish gills can result from exposure to elevated selenium. Field studies have found similar gill damage in fish exposed to selenium-laden effluent (Sorensen et al., 1982a,b, 1984) but the swelling of individual lamellae was not as severe and, because of the presence of other contaminants, a positive link to selenium exposure could not be made. Telangiectasia in fish gills is known to result from exposure to a variety of environmental contaminants (Meyers and Hendricks, 1985) and thus is not a specific bioindicator of selenium poisoning. Results of the present study support the observations of Sorensen et al. (1982a,b, 1984), who suggested that selenium was the cause of gill damage in environmentally exposed fish. However, other pathological changes found in the field studies, i.e., damage to liver, kidney, heart, and ovary, did not occur in the laboratory. This is likely due to different intake levels of selenium; fish in field studies consumed food items with 20–50 μg/g or more, whereas bluegill in the laboratory were fed only 5.1 μg/g.

About 60% of the secondary lamellae were severely dilated and most of the remaining ones showed some swelling, particularly at the base (Figs. 3–5). Externally, these

changes in structure suggest that efficient gas exchange was impossible due to restricted water flow through the gills. Within the lamellae, pools of blood in swelled sinuses would be less efficient at counter-current gas exchange than the normal condition of linear capillary flow (Holeton, 1980; Hughes, 1980). Reduced MCHC and competitive binding of selenium to hemoglobin would prevent saturation of erythrocytes and further decrease the amount of oxygen available to body tissues. Lemly (1982) found that in waterborne routes of exposure, most of the selenium in fish blood was associated with erythrocytes, suggesting that binding to hemoglobin or other cellular constituents occurs. Thus, reduced respiratory capacity from selenium exposure can be attributed to physical changes in gill tissue as well as changes in blood parameters that affect oxygen transport.

Winter Stress Syndrome and reduced survival

Although the respiratory capacity of the selenium-exposed fish appeared to be compromised, increased oxygen consumption occurred both in the warm- and cold-water treatment (Fig. 7). The possible imbalance between respiratory demands and respiratory capacity could have constituted a stress that resulted in reduced body condition and lipid content of fish in the cold-water treatment. Fish in the warm-water treatment continued to actively feed, which offset the energetic demands of increased oxygen consumption and reduced respiratory capacity. Body condition and total body lipid remained high in these fish, and were similar to the corresponding low-selenium control (Figs. 6 and 8). As water temperature declined in the cold-water treatment, bluegill reduced feeding and were consuming only a small fraction of the amount eaten by fish in warm-water exposures. A portion of the lipid depletion is attributable to natural reductions in feeding by bluegill during cold weather; lipid decreased by about 3% in the low selenium, cold-water control (Fig. 8). However, the degree of lipid depletion was much greater in selenium exposures, where reductions were 7–9%, indicating that selenium placed additional metabolic stress on these fish.

The differences in selenium content between the cold-water and warm-water selenium treatment probably resulted from changes in total body lipid rather than actual increases in concentration in the tissues. Selenium concentrations were similar on day 60 (Fig. 1), as was lipid content (Fig. 8). During the 60–120-day interval, weight, body condition, and lipid content fell in the cold-water treatment. Most selenium in fish tissue is associated with protein and blood-rich organs, not lipid (Cappon and Smith, 1982; Lemly, 1982). Therefore, large reductions in lipid could have occurred without appreciable changes in the total body burden of selenium. Selective reduction of lipid due to metabolic stress would have thus removed the 'dilution' effect of this tissue and caused selenium concentrations to appear to rise on a whole-body basis, which is how the samples were formulated and analyzed.

The combination of stress-related elevation in energy demand and reductions in feeding due to cold temperature and short photoperiod, leading to severe depletion of

stored body lipid, is herein given the name Winter Stress Syndrome. In the present study this syndrome caused bluegill to undergo an energetic drain that resulted in death of about one-third of the fish by day 120 (Fig. 9). The key element in this mortality was the influence of environment on behavior, i.e., reduced feeding as a result of lowered water temperature and shortened photoperiod. Mortality occurred when lipid content reached about 4.3% of total body weight, but was not significantly different than live fish taken from the tanks on scheduled sampling days. This finding indicates that most of the living fish were at a critical point for continued survival and that their level of feeding was barely keeping them alive. However, these fish did not die and in a natural setting water temperatures and feeding would increase after 2–4 months of cold weather, corresponding to days 120–180 of this study. Thus, the pattern of mortality that occured during the first 120 days probably gives an accurate indication of what would happen under field conditions.

Most of the mortality (87%) occurred during the first 120 days, which was within 60 days of the time water temperatures reached their lowest level (4°C). This suggests that the potential for Winter Stress Syndrome to kill young bluegill is just as great during a winter with 60 days of 4°C water as it is during one with 120 days of 4°C water. In the eastern US, winter water temperatures of impoundments where bluegill are an important species for recreational fishing are quite variable and depend on latitude, size, inflow conditions, and climatic fluctuations. However, it is reasonable to expect that many of these bodies of water would experience at least 60 days of 4°C water during an average winter. This is especially true for shallow littoral areas used for overwintering by young bluegill (Lemly, 1983). For these impoundments, annual differences in the severity and duration of cold weather should have little effect on the overall impact of Winter Stress Syndrome. However, substantial year-to-year differences could occur at locations that have milder climates and less than 60 days of 4°C water.

The two basic components of Winter Stress Syndrome, i.e., increased respiratory demands and lipid depletion, appear to be a generic set of physiological responses rather than a unique result of selenium exposure. For example, the syndrome can develop in bluegill because of parasite infestations and associated host tissue responses (Lemly and Esch, 1984). The pattern of increased respiratory demands and lipid depletion is identical regardless of whether the stressor is a parasite or a contaminant. In both cases, fish die when total body lipid falls to 3–6%. Lipid depletion is a normal occurrence for juvenile bluegill and other centrarchids that reduce feeding and become semi-dormant during cold weather (Lemly and Esch, 1984; Oliver et al., 1979; Toneys and Coble, 1980). However, the lipid content of young bluegill does not usually fall below about 7% and little mortality occurs (Lemly and Esch, 1984). Winter Stress Syndrome greatly accellerates the process of lipid depletion by adding to the energetic demand. Having been metabolically programmed by low water temperature, young bluegill do not respond to the demand with increased feeding; lipid stores necessary for overwintering are depleted and substantial mortality may follow.

Two types of stressors seem to have great potential to induce Winter Stress Syndrome in fish on a broad scale; parasites and water pollutants. Fish parasites do not often cause direct mortality of their hosts, however, many species are known to cause sublethal tissue pathology and associated metabolic effects (Davis, 1953; Amlacher, 1970; Ribelin and Migaki, 1975). These situations could develop into Winter Stress Syndrome if the host fish is a species or life stage that becomes semi-dormant in cold weather. Several categories of chemical contaminants are known to induce gill damage and respiratory stress similar to that observed for selenium in this study (Eller, 1975; Meyers and Hendricks, 1985). Falling water temperature may be all that is necessary to initiate the metabolic and physiologic changes that characterize Winter Stress Syndrome. Centrarchids and other warm-water fish species in the eastern US appear to be at high risk from these two types of stressors.

The implications of Winter Stress Syndrome are serious for juvenile bluegill and other species of fish that substantially reduce feeding during cold weather. Once water temperatures drop in the fall, a significant number of these fish could succumb to the stress syndrome within 60 days. In the southeastern US the critical period would occur in November, December, and January. Most mortality would likely take place during these three months. The present study and Lemly and Esch (1984) found 33% and 20% mortality in bluegill due to a single stressor; one was biological (parasite) and one was chemical (selenium). It is easy to envision a situation in which two or more of these would occur simultaneously, perhaps involving both biological and chemical stressors. The cumulative impact of multiple stessors and associated death of young fish could, in effect, offset reproduction. Winter Stress Syndrome can be a subtle, but important regulator of bluegill populations because of seasonal mortality in young-of-the-year and juveniles.

Implications for hazard assessment of selenium and other contaminants

Findings from previous studies which suggest that 5 μg/l selenium in water poses little threat to fish should be re-evaluated in the context of Winter Stress Syndrome. Waterborne concentrations of selenium in the 5 μg/l range are known to accumulate in fish food organisms to levels of 5 μg/g dry weight or higher (Besser et al., 1993; Cumbie and Van Horn, 1978), which equal or exceed the dietary concentrations fed in the present study. Winter Stress Syndrome has the potential to markedly lower the amount of selenium necessary to kill fish. Responses that would be sublethal for most of the year could become lethal during brief periods of cold-water temperature.

It has been concluded that reproductive success is the most sensitive indicator of selenium impacts on bluegill and other fish when simultaneous dietary intake and waterborne exposure are considered (Lemly, 1985a, 1985b; Lemly and Smith, 1987). This conclusion now bears revision because non-reproductive mortality from Winter Stress Syndrome can be an important, and perhaps the most significant component of effects near the low end of the toxicity threshold. Concentrations of 10–16 μg/g dry

weight dietary selenium and 5–10 μg/l waterborne selenium are probably necessary to cause reproductive failure of bluegill (Woock et al., 1987; Hermanutz et al., 1992; Coyle et al., 1993), yet only about half these amounts can be lethal to young-of-the-year and juveniles exposed to falling water temperatures (Fig. 9). Both reproductive and non-reproductive endpoints should be used to evaluate possible selenium problems in fish populations.

Several reservoirs in the southeastern US have experienced selenium accumulation and associated fishery problems as a result of aqueous discharges from coal-fired power plants (Cumbie and Van Horn, 1978; Garrett and Inman 1984; Gillespie and Baumann, 1986). Concentrations of selenium reached levels that caused acute toxicity and reproductive failure of bluegill and other centrarchids. Selenium inputs have been curtailed in some of these reservoirs and the fish populations are begining to recover, as evidenced by increasing reproductive success. However, recovery has been very slow even though waterborne and food-chain concentrations of selenium have dropped dramatically and are below thresholds that affect reproduction (Woock et al., 1987; Sorensen, 1988; Coyle et al., 1993; Lemly, 1993a,b). Winter Stress Syndrome may be operating to slow the recovery of fish populations in these reservoirs by reducing the overwinter survival of young-of-the-year. It is likely that seasonal mortality from Winter Stress Syndrome will be an important regulatory influence on bluegill and other centrarchids in these aquatic systems for many years.

Results of this study indicate that the current US national water quality criterion for selenium, 5.0 μg/l (US EPA, 1987), is not adequate to protect fish species that experience Winter Stress Syndrome. The adequacy of this criterion is also brought into question by several other recent field and laboratory studies which found that selenium can bioaccumulate to toxic levels in aquatic food chains when waterborne concentrations are in the 1.0–5.0 μg/l range (Barnum and Gilmer, 1988; Hoffman et al., 1990; Skorupa and Ohlendorf, 1991; Besser et al., 1993). Moreover, as little as 1.0 μg/l selenium may cause accumulation in fish and subsequent poisoning of aquatic birds and mammals that feed on them (Peterson and Nebeker, 1992). The true no-effect concentration of selenium for bluegill and other sensitive species of fish and wildlife is well below 5.0 μg/l; the US national criterion should be revised to reflect this fact.

The results of this study indicate that in order to fully assess the hazard of water pollutants to fish, laboratory tests must measure responses under changing environmental conditions. For example, laboratory protocols for studies of chemicals routinely specify that constant temperatures of 20–30°C be maintained when using warm-water fish (McKim, 1985; Petrocelli, 1985). Evaluating the role of mortality from Winter Stress Syndrome, which results from changing water temperature, cannot be done in this type of contaminant study. However, it may be critical to determine winter stress mortality in order to establish true no-effect concentrations and develop environmentally sound water quality criteria.

It is important to evaluate contaminants in relation to environmental stressors that

impinge on fish during only part of the year. Winter Stress Syndrome may occur for only a few weeks, yet 20–30% of a year class of fish could be eliminated. Field validation is one approach that has been used to evaluate results of laboratory studies and place them into an ecological context (Sanders, 1985). However, it may be difficult to establish cause–effect relationships and the results will be inaccurate if the validation studies do not last long enough to detect normal, seasonal changes in the organism. Laboratory studies that measure interactive effects of contaminants and other environmental stressors are critical to our understanding of contaminant threats to fish, particularly the early life stages.

Water temperature and water chemistry are generally recognized as being important determinants of contaminant toxicity to fish (Sprague, 1985). However, previous studies have focused on how these factors affect the speciation and uptake of chemicals, and toxicity thresholds, i.e., direct effects, rather than examining how seasonal changes in temperature modify the organism and, thereby, its sensitivity and response. Physiological studies have also concentrated on describing direct effects. Emphasis has been placed on determining mechanisms of toxicity and developing bioindicators of effects rather than evaluating to what extent the physiological changes that result from normal seasonal fluctuations in environmental conditions modify responses (Mehrle and Mayer, 1985). The present study combines both approaches by integrating measures that determine the metabolic status of the test organism – oxygen consumption and lipid content – into the experimental design. Consequently, the study yields information on how changes in environmental conditions affect the physiology of the organism and, in turn, the toxicity of selenium. The findings for selenium suggest that this approach should be used to re-evaluate the toxicity of many contaminants to young bluegill and other centrarchids. Winter Stress Syndrome could be an important, but as yet unkown mortality factor in many circumstances.

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